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Acyl-Acyl Carrier Protein Is a Donor of Fatty Acids in the NodA-Dependent Step in Biosynthesis of Lipochitin Oligosaccharides by Rhizobia

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NodA controls transfer of a fatty acid in the biosynthesis of lipochitin oligosaccharides by rhizobia. In an in vitro assay, we used de-N-acetylated chitin oligosaccharides substituted with an O-acetyl moiety as acyl acceptor substrates. We show that acyl-acyl carrier protein is used as a donor in NodA-directed fatty acid transfer.

Rhizobia, bacteria of the genera *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium*, produce lipochitin oligosaccharides (LCOs) as signal molecules involved in root nodulation. The NodA, NodB, and NodC proteins are essential for the biosynthesis of the core of LCOs. NodC is presumed to be a glucosaminyl-transferase, which synthesizes the chitin oligosaccharide backbone by using UDP-*N*-acetylglucosamine as the substrate (5, 7, 15). The nonreducing terminal saccharide residue is de-N-acetylated by NodB (6), after which NodA is involved in the transfer of a fatty acid to the free amine group (1, 12). Of these proteins, the biochemical function of NodA is the least understood. Little is known about acyltransferases in prokaryotes. The use of acyl-acyl carrier protein (acyl-ACP) as a donor in transacylation in the biosynthesis of phospholipids has been reported (8, 11). In this paper, we address the question of which fatty acid donor is used in the transacylation reaction directed by NodA. We test the use of free fatty acids and acyl-ACP as an acyl donor in the transacylation reaction determined by NodA. We show that acyl-ACP is used as a fatty acid donor in LCO biosynthesis, whereas free fatty acids cannot be incorporated.

In vitro analysis of NodA function with radiolabeled acceptor substrate. Data reported by Bloemberg et al. (2) show that the preferred substrate for the addition of the O-acetyl group by NodL is a de-N-acetylated chitin oligosaccharide. This indicates that, during biosynthesis, NodL adds the O-acetyl group to the oligosaccharide backbone before acyl transfer directed by NodA. We therefore decided to test O-acetylated de-N-acetylated chitin tetrasaccharides as acceptors in an NodA-dependent in vitro acyltransferase reaction.

De-N-acetylated chitin tetrasaccharides were synthesized as described previously (17) and treated with partially purified NodL (NodL was purified up to the ammonium sulfate precipitation step as described by Bloemberg et al. [3]) in the presence of [^{14}C]acetyl-coenzyme A (CoA) overnight at 30°C. Thin-layer chromatography (TLC) analysis was performed with an amino-silica gel plate, which was developed in 65% acetonitrile and analyzed with a phosphorimaging system. The results show that the reaction yielded ^{14}C-O-acetylated derivatives of de-N-acetylated chitin tetrasaccharides (Fig. 1A). The other spots that are detected after TLC analysis could be due to breakdown of the oligosaccharide, yielding mono-, di-, and trisaccharides. One of the spots is probably free acetate, resulting from an aspecific release of acetate by NodL, as has been described previously (3). Unused acetyl-CoA was not detected, presumably as a result of hydrolysis.

Because it has not been possible to obtain active purified NodA, we chose to use an in vitro transacylation assay described by Röhrig et al. (12). The assay uses 1.0 ml of a preparation of sonicated *Rhizobium* cells (total optical density at 660 nm, 30) (12). ^{14}C-labeled O-acetylated de-N-acetylated chitin tetrasaccharides were added (total amount of radioactivity, 0.3 nCi), and the reaction was performed for 3 h at 30°C. Extraction with 0.6 volume of water-saturated *n*-butanol separated the synthesized LCOs from the O-acetylated derivatives of de-N-acetylated chitin tetrasaccharides which were used as acyl acceptor substrates. The *n*-butanol was dried, and the pellet was dissolved in 50 μl of acetonitrile-water (1:1 [vol/vol]). After centrifugation, the supernatant fluid was dried and dissolved in 10 μl of water-saturated *n*-butanol. The sample was spotted onto a TLC plate of C_{18}-bonded silica gel that was developed in acetonitrile-water (1:1 [vol/vol]). As a reference sample, LCOs purified from *Rhizobium leguminosarum* bv. viciae RBL5045.pIJ1089 were used, since this strain produces LCOs which are O acetylated and which have a tetrasaccharide backbone.

Several strains of *Rhizobium* were tested in the assay described above (see legend to Fig. 3). A comparison of the radioactive spots detected after TLC analysis showed that the most efficient acylation was achieved with *Rhizobium meliloti* (data not shown). For subsequent experiments described in this paper, four strains were used: (i) ISV1501, which contains a Tn*5* insertion in *nodB* (12) (ii) ISV1502, which contains a Tn*5* insertion in *nodA* (12); (iii) GMI766, which contains a deletion in the Sym plasmid, including all of the *nod*, *nol*, and *noe* genes (16); and (iv) GMI766 harboring plasmid pMP4173, which contains *nodA* of *Bradyrhizobium* sp. strain ANU289 and *nodD* of *R. leguminosarum* bv. viciae (Fig. 2). We use NodA of *Bradyrhizobium* sp. strain ANU289 because it is not specialized for the transfer of polyunsaturated fatty acids, in contrast to NodA of *R. meliloti* (4, 10).

TLC analysis showed that the extracts of the *nodA*-containing strains ISV1501 and GMI766.pMP4173 produce radioactive spots, which have an R_f value comparable to those from the reference LCOs (Fig. 3). The spots presumably represent

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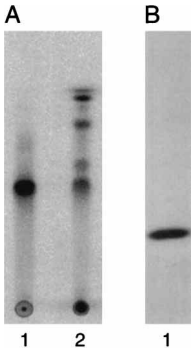

FIG. 1. Analysis of acyl acceptor and donor substrates. (A) TLC analysis of de-N-acetylated chitin tetrasaccharides which are O acetylated by NodL and [^{14}C]acetyl-CoA. The following samples were treated with NodL: lane 1, de-N-acetylated chitin tetrasaccharides (12); lane 2, de-N-acetylated chitin tetrasaccharides obtained by organic synthesis (17). (B) Native polyacrylamide gel electrophoresis analysis of synthesized [^{3}H]palmitoyl-ACP.

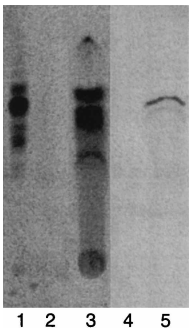

FIG. 3. TLC analysis of a transacylation assay with ^{14}C-labeled O-acetylated de-N-acetylated chitin tetrasaccharides as the acceptor substrate. Radioactivity was detected with a phosphorimaging system. Lanes: 1, reference LCOs from strain RBL5045.pIJ1089 (14); 2, extracts from *R. meliloti* ISV1502 (*nodA*::Tn*5*); 3, extracts from *R. meliloti* ISV1501 (*nodB*::Tn*5*); 4, extracts from *R. meliloti* GMI766 (Δ*nod*); 5, extracts from *R. meliloti* GMI766.pMP4173 (*nodA* from *Bradyrhizobium*). Also tested were extracts from *R. leguminosarum* bv. viciae 248 and RBL5560, *R. leguminosarum* bv. trifolii ANU843, and *R. leguminosarum* bv. phaseoli 8400, which had very poor transacylation activities (data not shown).

LCOs with different fatty acids. Extracts of strains not containing *nodA* did not produce such spots on TLC.

Using strain ISV1501, Röhrig et al. (12) showed that NodA of *R. meliloti* is involved in the transfer of fatty acids to de-N-acetylated chitin tetrasaccharides. Using another *R. meliloti* strain, Atkinson et al. (1) showed that sulfated oligosaccharides are acyl acceptor substrates. Our data add O-acetylated de-N-acetylated chitin tetrasaccharides to the list of possible acceptor substrates in NodA-dependent transacylation.

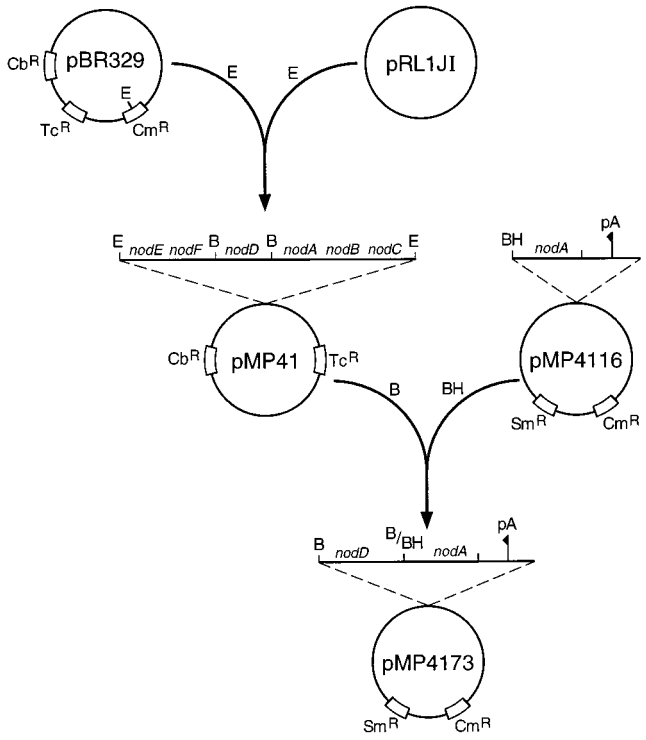

FIG. 2. Construction of plasmids. Plasmid pMP4173 is a derivative of the *Bradyrhizobium* sp. strain ANU289 *nodA*-containing plasmid pMP4116 (10). In this plasmid, *nodD* was cloned, because NodD protein is needed for transcriptional activation of *nodA*. The *nodD* gene of *R. leguminosarum* bv. viciae was obtained from plasmid pMP41, a derivative of pBR329 containing a 6.4-kb *Eco*RI fragment of the plasmid pRL1JI, which encodes *nodABCDEF*. The *nodD* gene was excised from plasmid pMP41 with *Bcl*I restriction sites and ligated into pMP4116 restricted with *Bam*HI. B, *Bcl*I; BH, *Bam*HI; E, *Eco*RI; Cb^r, carbenicillin resistance; Cm^r, chloramphenicol resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

In vitro analysis of NodA with radiolabeled fatty acids coupled to ACP as the donor. ACP of *Escherichia coli* was acylated with acyl-ACP synthetase and [^{3}H]palmitate (specific activity, 50 Ci/mmol) and purified as described by Ritsema et al. (9). One single band of radiolabeled protein was seen after electrophoresis on a 17.5% native polyacrylamide gel (Fig. 1B). An amount of 3.5 nCi of acyl-ACP was added to a preparation of 1 ml of sonicated cells. Nonradioactive O-acetylated de-N-acetylated chitin tetrasaccharide was prepared by the method described for the radioactive acceptor, with nonradioactive acetyl-CoA. The acceptor was added at the same amount used in assays with radioactive acceptor. With cellular extracts of the *nodA*-harboring strains ISV1501 (*nodB*::Tn*5*) and GMI766.pMP4173 (*nodA* from *Bradyrhizobium*) we could detect a spot on a C_{18}-silica TLC plate that was not present when cellular extracts of strains that did not harbor *nodA* were used (Fig. 4A and results not shown). The NodA-dependent spot has an R_f value that is expected for an LCO containing a $C_{16:0}$ fatty acid and an O-acetyl group (13). When O-acetylated de-N-acetylated chitin tetrasaccharides were not added, spots with a comparable R_f value could not be detected (Fig. 4C). Because we hereby have shown that not only do the spots contain palmitate, the O-acetylated de-N-acetylated chitin tetrasaccharides are necessary to produce them, we conclude that the spots obtained are indeed LCOs.

When instead of acyl-ACP the same amount of free fatty acids was used, no spots with an R_f value similar to that of the LCOs could be detected (Fig. 4B). We therefore conclude that free fatty acids cannot be used for the NodA-dependent transacylation. This shows that the acyl moiety of acyl-ACP was not released by hydrolysis prior to transacylation.

The use of crude cell extracts for our in vitro transacylation reaction implies that the radiolabeled acyl-ACP donor substrate was able to compete successfully with putative unlabeled acyl donors present in the extract. Considering the small amount of radioactive $C_{16:0}$ fatty acids that we added in the form of acyl-ACP (approximately 1 pmol), the results indicate that acyl-ACP is an efficient acyl donor in the acyltransferase reaction mediated by NodA. Therefore, putative other donors

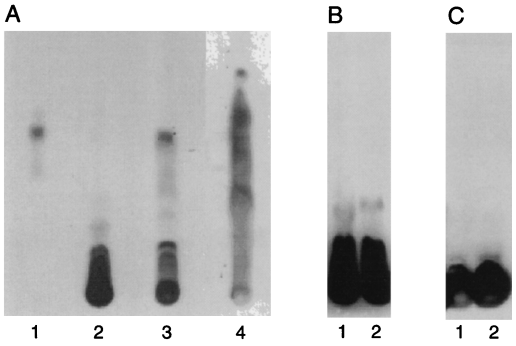

FIG. 4. (A) TLC analysis of a transacylation assay with [^{3}H]palmitate-ACP as the donor substrate. O-acetylated de-N-acetylated chitin tetrasaccharides were used as the acyl acceptor substrate. Radioactivity was detected by autoradiography. Enhancement of the signal was obtained by spraying TLC plates with En^{3}Hance (DuPont). Lanes: 1, reference LCOs from strain RBL5045.pIJ1089; 2, extracts from ISV1502 (*nodA*::Tn*5*); 3, extracts from ISV1501 (*nodB*::Tn*5*); 4, extracts from ISV1501 (*nodB*::Tn*5*) with a radiolabeled acceptor (Fig. 3, lane 3). (B) TLC analysis of a transacylation reaction with free [^{3}H]palmitate. Lanes: 1, extracts from ISV1502 (*nodA*::Tn*5*); 2, extracts from ISV1501 (*nodB*::Tn*5*). (C) TLC analysis of a transacylation reaction without the use of O-acetylated de-N-acetylated chitin tetrasaccharides as the acceptor substrate. [^{3}H]palmitate-ACP was used as the acyl donor substrate. Lanes: 1, extracts from ISV1502 (*nodA*::Tn*5*); 2, extracts from ISV1501 (*nodB*::Tn*5*).

of fatty acids in the cell that are abundantly present in the extracts used, such as phospholipids, can be ruled out as competitive donor substrates in LCO biosynthesis. Our results therefore strongly suggest that acyl-ACP in vivo is used as a donor of fatty acids during the NodA-dependent transacylation in LCO biosynthesis.

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